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Manuscript Title

Methylmercury and long chain polyunsaturated fatty acids are associated with immune dysregulation in young adults from the Seychelles Child Development Study.

Authors

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Abstract

BACKGROUND: Exposure to the environmental toxicant mercury (Hg) has been associated with immune dysregulation, including autoimmune disease, but few human studies have examined methylmercury (MeHg) exposure from fish consumption.

OBJECTIVES: We examined associations between MeHg exposure and biological markers of autoimmunity and inflammation while adjusting for long chain polyunsaturated fatty acids (LCPUFA).

METHOD: At age 19 years, hair total Hg (Y19Hg), LCPUFA status, a panel of 13 antinuclear antibodies (ANA), total serum immunoglobulins (Ig) IgG, IgA, and IgM and serum markers of inflammation (IL-1, IL-2, IL-6, IL-10, C-reactive protein (CRP), IFN-γ, TNF-α) were measured in the Seychelles Child Development Study (SCDS) Main Cohort (n=497). Multivariable regression models investigated the association between Y19Hg and biomarkers, adjusting for prenatal total hair Hg (MatHg) and other relevant covariates, and with and without adjustment for LCPUFA.

RESULTS: With each 1 ppm increase in Y19Hg (mean 10.23 (SD 6.02) ppm) we observed a 4% increased odds in a positive Combined ANA following adjustment for the n6:n3 LCPUFA ratio (β = 0.036, 95%; CI: 0.001, 0.073). IgM was negatively associated with Y19Hg (β = -0.016, 95%CI: -0.016, -0.002) in models adjusted for n-3, n-6 LCPUFA and when separately adjusted for the n-6:n-3 LCPUFA ratio. No associations were observed with MatHg. Total n-3 LCPUFA status was associated with reduced odds of a positive anti-ribonuclear protein (RNP) A. The n-3 LCPUFA were negatively associated with IL-6, IL-10, CRP, IFN-γ, TNF-α and positively with TNF-α:IL-10. There were positive associations between the n-6:n-3 ratio and IL-6, IL-10, CRP, IFN-γ, TNF-α and a negative association with TNF-α:IL-10.

DISCUSSION:

The Y19Hg exposure was associated with higher ANA and lower IgM albeit only following adjustment for the n-3 LCPUFA or the n-6:n-3 LCPUFA ratio. The clinical significance of these findings is unclear, but warrant follow up at an older age to determine any relationship to the onset of autoimmune disease.

KEY WORDS: autoimmunity; methylmercury; autoantibody; cytokine; immunoglobulin

Introduction

Exposure to the ubiquitous environmental toxicant mercury (Hg) has been associated with immune dysregulation including autoimmune disease (Blossom & Gilbert, 2018). It is proposed that Hg exposure, in combination with genetic predisposition, may result in autoimmune disease development or exacerbation (Silbergeld *et al.*, 2005), albeit nearly all this evidence is derived from experimental animal studies with inorganic Hg exposure and evidence from human studies is lacking (Crowe *et al.*, 2017; Bjorklund *et al.*, 2017). Humans are primarily exposed to organic Hg following consumption of fish, which bio-accumulate organic methylmercury (MeHg) from their environment. If Hg is associated with autoimmune disease in people, it would be a major public health concern as fish are an important source of protein in many populations globally.

Immunotoxic effects of Hg have been observed in murine models where exposure to Hg (either organic or inorganic) results in the expression of autoimmune markers including anti-nuclear antibodies (ANA), anti-nucleolar antibodies (ANoA) and anti-chromatin (ACA); (Crowe *et al*., 2017; Pollard *et al.*, 2019). In humans, several studies investigating occupational Hg exposure in artisanal gold mining communities have reported elevated titres of ANA and ANoA along with elevated concentrations of inflammatory markers (Il-1β, TNF-α and IFN-γ) (Silva *et al*., 2004; Alves *et al*., 2006; Nyland *et al*., 2011; Motts *et al*., 2014). Others, however, have observed no association between Hg and biomarkers of immune dysfunction (Barregard *et al.,* 1997; Ellingsen *et al*., 2000; Alves *et al*., 2006; Sánchez Rodríguez *et al.,* 2015). Analysis of the U.S. National Health and Nutrition Examination Survey (NHANES) data has identified associations, in women, between higher blood Hg concentrations and ANA positivity (Somers *et al.,* 2015) as well as between Hg and higher concentrations of thyroid autoantibodies (Gallagher and Meliker, 2018). In a high fish consuming cohort from the Amazonian region, MeHg exposure was associated with higher IL-6, IFN-γ, IL-4 and IL-17 cytokine concentrations (Nyland *et al.,* 2011), but other studies have observed no association (Monastero *et al.,* 2017). The majority of research to-date has investigated concurrent Hg exposure with one study reporting an inverse association between prenatal MeHg exposure at 28 weeks gestation and immune markers (McSorley *et al.,* 2018).

Associations between markers of autoimmunity and MeHg exposure in populations with high fish consumption have not been widely investigated. An examination of prenatal and postnatal MeHg exposure and total serum IgG and IgM concentrations in a fish-eating cohort from the Faroe Islands reported significant associations with postnatal MeHg exposure at age 7 years and both IgG and IgM concentrations (Osuna *et al.,* 2014). Conflicting with this

finding, no association was observed between concurrent MeHg and markers of autoimmunity within a seafood consuming population from Long Island, New York (Monastero *et al.,* 2017). Overall, the interpretation of existing research is hampered by differences in sources of MeHg exposure, varying sample size and the presence in some studies of malaria which affects immunity (Sánchez Rodríguez *et al.,* 2015). Thus, large population based studies are required to fully elucidate any potential impact of Hg exposure, particularly that of MeHg from fish consumption, in the development of autoimmune disease (Pollard *et al.,* 2010). Adding to the complexity, fish are a rich source of the long chain polyunsaturated fatty acids (LCPUFA), predominately n-3 LCPUFA, which have anti-inflammatory properties and are associated with a reduction of circulating inflammatory markers (Calder, 2015). Therefore, when investigating immunotoxic effects of MeHg, research should also consider the potential beneficial effects of LCPUFA on immune function.

The fish-eating cohort of 19 year olds from the Seychelles Child Development Study (SCDS) have an average consumption of 7 fish meals per week and a MeHg exposure approximately 10 times the levels in the United States (van Wijngaarden *et al.,* 2017). Using this cohort, we investigated whether MeHg exposure from fish consumption (prenatal and concurrent exposure) was associated with markers of autoimmunity and inflammation. It was hypothesised that both prenatal and concurrent MeHg exposure would be associated with markers of autoimmunity and inflammation and that n-3 LCPUFA would mitigate these associations.

Methods

Study design

The SCDS enrolled 779 pregnant women during 1989-1990 as the 'Main cohort' to investigate associations between prenatal MeHg exposure and child neurodevelopment. At the 6-month time point, data for 39 mother-child pairs were excluded owing to mother's illness during pregnancy, insufficient maternal hair to recapitulate prenatal MeHg exposure, twin births or children born with conditions known to affect neurodevelopment (e.g. prematurity, severe perinatal illness, closed head trauma with loss of consciousness, encephalitis or meningitis). Subsequent exclusions through age 19 years for epilepsy, head trauma or meningitis resulted in the removal of 56 additional participants leaving a total of 684 participants at 19 years of age for analysis.

A total of 530 serum samples from the participants at 19 years were collected and stored at -80°. Data for 497 participants' immune markers were used in the present study owing to inadequate serum volume for analysis in 29 samples and 4 specimens of immune markers could not be matched with study ID numbers. The study protocol was reviewed and approved by the Seychelles Ethics Board and the Research Subjects Review Board at the University of Rochester.

Immunology testing

Stored serum samples were thawed and analysed at Ulster University. Inflammatory cytokines were measured using the electrochemiluminescence based Meso Scale Discovery (MSD) multiplex assay (Meso Scale Diagnostics, LLC.) and included interleukin (IL)-1β, IL-2, IL-6, IL-10, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). All cytokines are reported in pg/ml. Inter and intra assay cytokine CVs were IL-1β (28.61%; <28%), IL-2 (30.67%; <24%), IL-6 (25.81%; <23%) , IL-10 (23.1%; <33%), IFN-γ (21.87%;<32%) and TNF-α (12.11%; <20%). CRP (mg/L), a marker of acute inflammation, was measured by an ultra-sensitive diagnostic kit (Werfen Ltd. England) using the iLab 650 Clinical Chemistry Analyzer and had an inter-assay CV of 16.37% and an intra assay CV of <12%. Cytokine measurements below the lower limited of detection (LLOD), as determined by the standard curve for each cytokine individually, was assigned a value of LOD/√2 for statistical analysis. Serum immunoglobulin-A (IgA), IgG and IgM (g/L) were measured by ELISA (Thermo Fisher, UK) at the Immunology Laboratory, Royal Victoria Hospital Belfast.

ANA status was screened for using the BioPlex ANA fully automated multiplex system (BioRad, UK) which has good concordance with comparative methods (Sohn & Khan, 2014). Screening of the samples by indirect immunofluorescence characterized the presence or absence of specific antinuclear antibodies (ANA). All samples were analysed for the quantitative detection of anti-dsDNA and the semi-quantification in antibody of anti-ribosomal P, anti-chromatin, anti-Ro52, anti-Ro60, anti-La, anti-Sm, anti Sm/RNP, anti-RNP A, anti-RNP 68, anti-Scl-70, anti-Jo-1 and anti-centromere B. The ANA screen is reported as negative if the results for all 13 autoantibodies are negative. Conversely, if any of the 13 autoantibodies is positive, we report a positive ANA screen and the Antibody Index (AI) of individual antibodies. The AI is an arbitrary unit defined by the manufacturer when no official standards are available. Anti-dsDNA antibody is calibrated against World Health Organization Wo/80 standard and expressed in terms of IU/mL with values ≥4 IU/mL reported as positive. All other antibody results are semi-quantitative, expressed in terms of AI, and values ≥1.0 AI are taken as positive.

Methylmercury exposure

Prenatal MeHg exposure was determined using maternal hair samples collected either during pregnancy, at delivery, or at the 6-month enrolment (Myers *et al.,* 1995). MatHg was measured as total Hg from these hair samples by cold vapour atomic absorption spectroscopy where the closest centimetre to the scalp represents the most recent exposure of one month. Concurrent postnatal exposure at 19 years of age (Y19Hg) was measured using the same approach in a 1 cm length of each participant's hair taken at time of testing. All Hg results are presented as MeHg are total Hg (THg) based on the assumption that ~80% of THg in hair is MeHg within the Seychelles population (Cernichiaria *et al.,* 1995).

Fatty acid analysis

Plasma phospholipids were measured at 19 years as outlined previously (van Wijngaarden *et al.,* 2013). In brief, total lipids were extracted from plasma samples, using a modified method of Folch *et al.,* (1957). A solid phase extraction using an NH2 cartridge system conditioned with chloroform and followed by a series of solvent elution's was used to isolate phospholipids. Absolute amounts of LCPUFA were determined using gas chromatography mass spectrometry (GCMS) as described previously (Bonham *et al.,* 2008) and included linoleic acid (LA, C18:2 n-6), α-linolenic acid (ALA, C18:3 n-3), arachidonic acid (AA, C20:4 n-6), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). Results were presented as mg/mL to indicate physiologic quantities. Total n-6 (mg/ml) was calculated by the addition of LA and AA concentrations, and ALA, EPA and DHA were summed to calculate total n-3 (mg/ml). The n-6:n-3 ratio was calculated.

Statistical analysis

Descriptive statistics summarised the distributions of MeHg, serum cytokines, immunoglobulins, autoantibodies and covariates. Cytokine and immunoglobulin measurements were log transformed for the linear regression models as these measures were extremely right skewed. Clinically elevated concentrations of individual ANA markers were uncommon, perhaps because of the relatively young age of the 19-year study participants. For 11 of the 13 ANA markers, >95% of the ANA values were below the LLOD. Therefore, these ANA variables were not analyzed as individual markers. Instead, we designed a combined dichotomous ANA variable called 'Combined ANA' that was calculated based on being within or above reference range for one of more of the 13 measured ANA markers. Some 56% of the subjects met this criterion. In addition, because of their larger number of measurable values, Anti-dsDNA and Anti-RNP A were analyzed as both dichotomous (<LOD or LOD+) and categorical (<LOD, LOD-ref, >ref) individual markers.

Associations between serum cytokines, immunoglobulins, autoantibodies and pre- and postnatal MeHg exposure were examined through linear (cytokines and immunoglobulins) and logistic (autoantibodies) regression models. Covariates, known or suspected to be associated with (subclinical) autoimmunity or inflammation, included in the analysis were maternal age, child sex, socioeconomic status (SES) and obesity. Maternal socioeconomic status was measured using the Hollingshead Social Status Index modified for the use of employment codes relevant to the Republic of Seychelles (Davidson *et al.,* 1998; Kobrosly *et al.,* 2011). Models that included MatHg adjusted for maternal age at birth, maternal socioeconomic status (SES) and child sex. Models that included postnatal Y19Hg adjusted for child sex and waist circumference (WC) at 19 years as a proxy for abdominal obesity. Consistent with previous cross-sectional analyses of postnatal exposure in the SCDS (van Wijngaarden *et al.,* 2013), postnatal Hg models also adjusted for prenatal MeHg exposure measured in maternal hair. We additionally controlled for 19-year LCPUFA status in separate models to evaluate the possibility that adverse associations may be missed or underestimated due to uncontrolled confounding by the immunomodulatory effect of LCPUFA. In separate models we also included interactions of child sex with both MatHg and Y19Hg to assess whether males or females are more susceptible to the effects of MeHg exposure. Following analysis, we found no evidence for interactions; therefore, we report results from the corresponding models without this interaction.

All statistical analyses were performed using R, Version 3.5.1. Statistical significance in all analyses was determined using a two-sided approach $\alpha=0.05$. Regression model assumptions were checked using standard methods (Weisberg 2005). If violated, we consider transforming the outcome or fitting nonlinear additive models (Hastie and Tibshirani 1990). Results were evaluated for extreme outliers and unduly influential points. All values presented in tables are log transformed.

Results

Year 19 demographic characteristics for participants are displayed in Table 1. Immune markers for a total of 497 mother-child pairs were analysed, consisting of 268 females and 229 males. Mean (SD) MatHg and Y19Hg was 6.84 (4.55) and 10.23 (6.02) ppm, respectively. An average (SD) of 7.22 (3.66) fish meals per week were consumed by participants who had a mean (SD) n-6:n-3 LCPUFA ratio of 3.81 (1.97).

MeHg, LCPUFA, and anti-nuclear antibodies

Regression analyses for covariate adjusted associations between MeHg exposure and immunologic markers are presented in Table 2. The Y19Hg was significantly associated with a higher Combined ANA (β = 0.036, 95%; CI: 0.001, 0.073) where for each 1 ppm increase in Y19Hg we observed a 4% increased odds for a positive Combined ANA following adjustment for the n-6:n-3 LCPUFA ratio. MatHg was not significantly associated with a change in odds for a positive Combined ANA. Separately, a significant association was observed between the anti-RNP A and n-3 LCPUFA (Table 3). No significant association was found between n-6 LCPUFA and ANA or any immune marker.

MeHg, LCPUFA, and immunoglobulins (Ig)

Y19Hg was negatively associated with IgM in the models adjusted for n-3 LCPUFA (β = -0.009, 95%CI: -0.016, -0.002) and when separately adjusted for the n6:n3 LCPUFA ratio (β = -0.009, 95%CI: -0.016, -0.001) (Table 2). MatHg was not associated with any Ig. None of the MeHg metrics were associated with IgA. No significant associations were found with n-3 LCPUFA, n-6 LCPUFA and the n6:n3 LCPUFA ratio and IgG.

MeHg, LCPUFA, and cytokines and CRP

Significant positive associations were observed between Y19Hg and CRP in the models that adjusted for n-3 and n-6 LCPUFA (β = 0.031, 95%CI:0.007, 0.054) and the n-3:n-6 ratio (β = 0.031, 95%CI:0.007, 0.054) (Table 2). Y19Hg was significantly associated with IL-10 in the model that adjusted for n-3 and n-6 LCPUFA (β = 0.016, 95%CI:0.002, 0.030) and in the model which adjusted for the n-3:n-6 ratio (β = 0.016, 95%CI:0.002, 0.031). A statistically significant association was observed between Y19Hg and the TNFα:IL-10 ratio in the model adjusted for n-3 and n-6 LCPUFA (β = -0.016, 95%CI: -0.027, -0.005) and in the model which adjusted for the n-3:n-6 LCPUFA ratio (β = -0.016, 95%CI: -0.027, -0.005). MatHg was not associated with any of the measured cytokines or CRP.

There were significant negative associations between n-3 LCPUFA and CRP, IFN-γ, TNF-α, IL-6 and IL-10 (Table 3). A positive association was observed between n-3 LCPUFA and the TNFα:IL-10 ratio. Statistically significant positive associations were found between the n-6:n-3 ratio and CRP, IFN-γ, TNF-α, IL-6 and IL-10 and a significant negative association with the TNFα:IL-10 ratio. No significant associations were found between n-6 PUFA and any of the cytokines.

Covariates

Females had significantly greater levels of Combined ANA, anti-dsDNA, CRP and IgM in all models. Female had significantly lower levels of TNF-α in all models. Higher levels of maternal SES was significantly associated with increased IgA concentrations without adjustment for LCPUFA (β = 0.003, p = 0.006). Larger values of Year 19 WC was significantly associated with positive IL-6 concentrations (β = 0.022, p < 0.0001) and positive CRP concentrations (β = 0.047, p < 0.0001) in all models.

Discussion

Evidence from animal models suggests a potential link between Hg exposure and the pathogenesis of autoimmune disease. Less, however, is known in human populations especially with respect to MeHg exposure through fish consumption. In this high fish-eating population from the SCDS, we found that current MeHg exposure at 19-year of age was associated with a novel measure of ANA (Combined ANA) but only following adjustment for the n-6:n-3 LCPUFA ratio. Furthermore, Y19Hg was associated with lower IgM, higher CRP, higher IL10 and a lower TNFα:IL10 ratio. Prenatal Hg exposure was not associated with any specific marker of ANA, cytokines or Ig. Although current MeHg exposure at 19 years was associated with higher odds of having a higher ANA combined score the clinical significance of these findings is unclear and further research is warranted to determine if these associations precede autoimmune disease development. Total n-3 LCPUFA was associated with lower anti-RNP A, ANA and overall a more anti-inflammatory profile supporting the well-known benefits of n-3 LCPUFA in regulating the immune system (Calder *et al.,* 2015).

In this cohort, hair Hg concentrations were on average 10 times those reported from the USA (Davidson *et al.,* 1998; Myers *et al.,* 2003; van Wijngaarden *et al.,* 2017). At 19 years, this cohort reported an average consumption of 7 fish meals per week which correlated with Hg supporting the evidence that fish consumption is a significant predictor of MeHg exposure in humans (Schober *et al.,* 2003; Clark *et al.,* 2007; Bjermo *et al.,* 2013; Somers *et al.,* 2015). The higher exposure of Hg in the 19 year olds may, in part, explain why the associations between MeHg and ANA were only evident at this time point following adjustment for the n6:n3 LCPUFA ratio. It is also plausible that the measure of MeHg exposure at 19 years reflects better than MatHg exposure what is happening systemically in the blood sample taken at the same timepoint.

Previous research by our group has emphasised the importance of n-3 LCPUFA, obtained primarily in the diet through fish consumption, in mitigating any potential effects of MeHg

(Strain *et al.,* 2015; Strain *et al.,* 2008). The National Health and Nutrition Examination Survey (NHANES) investigated young females with hair Hg of 0.22ppm and blood Hg of 0.944 ug/L and, similar to our results, reported associations between ANA positivity and a high titre of ANA positively in models which also adjusted for n-3 LCPUFA (Somers *et al.,* 2015). Associations between Hg exposure and ANA have been reported in gold mining communities (Aves *et al.,* 2006, Gardner *et al.,* 2010) and to a lesser extent in a high fish-eating riverine community in Amazonian Brazil (Silva *et al.,* 2004, Nyland *et al.,* 2011). Within these studies, malaria infections are suggested to add to the strength of the association between Hg exposure and ANA. Furthermore, exposure to Hg is associated with dysregulation of inflammatory cytokines and cellular oxidative stress proteins which authors suggest contributes to the immunotoxicity of Hg (Motts *et al.,* 2014). Contrary to these findings, analysis in an American Sioux Tribe, who regularly consume fish from a river known to be ubiquitously contaminated with Hg showed that some 30% of individuals were positive for ANA, however there was no overall association between blood Hg values and ANA (Ong *et al.,* 2014). Within their analysis these workers did not adjust for n-3 LCPUFA which may have negatively confounded any associations with Hg (Budtz-Jorgensen *et al.,* 2007). Similarity, in a gold mining population in the Andes, Columbia, no difference in ANA status was observed between those exposed to Hg compared to those not exposed (Sanchez-Rodriguez *et al.,* 2015). Whilst they did adjust for estimated Hg intake from fish consumption it would have been interesting to see if adjustment for biological status of n-3 LCPUFA would have revealed an association given that there was a higher consumption of fish in those exposed to Hg compared to the non-exposed group. A pilot study of a fish-eating cohort from Long Island, USA also found no association between Hg and the expression of genes known to be involved in autoimmunity; however, they reported, owing to small sample size, that they did not control for n-3 LCPUFA in the analysis. Taken together, those studies that have investigated MeHg exposure from fish consumption, and have not adjusted for n-3 LCPUFA in the statistical models, have found no associations with markers of autoimmunity whereas those that have controlled for n-3 LCPUFA have reported associations, albeit the magnitude of effect appears to be related to the source of Hg (gold mining or from fish consumption) as well as other confounders including infections. The Seychelles population is not affected by malaria and has a primary route of MeHg exposure through fish consumption; therefore, the findings reported here support the existing literature that the benefits of LCPUFA from fish consumption outweigh any adverse effect of MeHg on health outcomes including autoimmunity.

The focus of this paper is MeHg exposure through fish consumption. Nevertheless, it is important to recognise that research to date, in murine models, has shown a stronger

association between inorganic Hg exposure and the development of autoimmune type responses (Crowe *et al*., 2018). Differences in intracellular diffusion and biodistribution of the two forms of Hg may explain why inorganic Hg is more strongly associated with the development of autoimmunity (Bjorklund *et al*., 2017; Pollard *et al*., 2019). MeHg is proposed to have a delayed and less inflammatory response without the development of immune complexes whereas inorganic Hg is associated with renal damage (Crowe *et al*., 2017).

The exact mechanisms involved in Hg-associated autoimmunity remains elusive with some suggestions that Hg may contribute to the stimulation and survival of autoreactive immune cells due to its ability to disrupt self-antigen presentation, functional B-cell signalling, effective class switching and the deletion of autoreactive immune cell clones (Crowe *et al*., 2017; Khan & Wang, 2018; Pollard *et al*, 2018). Hg may also contribute to the stimulation of an autoimmune response due to its ability to simulate the innate and adaptive response (Pollard et al, 2018). One potential mechanism by which Hg exposure could lead to autoimmunity is through Hg induced tissue damage resulting in the release of damage associated molecular patterns (DAMPs) and/or modified DAMPs with the subsequent activation of a local innate immune response alongside the activation of autoreactive B & T cells in the lymph node to illicit an autoimmune adaptive response (Pollard *et al*., 2018). Hg may also contribute to autoreactive B cell clone survival as it has been shown to disrupt B cell receptor signalling in immature B cells by targeting the tyrosine kinase protein, Lyn (Gill *et al*., 2017). Furthermore, disruption of BCR signalling mechanisms in immature B cells may disrupt negative selection of self-reactive clones and Ig class switching resulting in the loss of self-tolerance and the production of autoreactive B cells (Gill *et al.*, 2017).

In the current study, higher Y19Hg was significantly associated with lower IgM which may suggest an alteration in class switching. IgM is the first antibody to respond to an antigen or self-antigen and is involved in enhanced antibody response and activation of the complement cascade resulting in the inflammatory response. IgM plays a regulatory role in subsequent immune response development, thereby accelerating the production of high-affinity IgG. Lower IgM has been associated with decreased T helper activity, increased isotype-specific suppressor T cell activity, and intrinsic B cell defects (Louis & Gupta, 2014). The association between lower IgM and higher Hg in this study could be indicative of a dysfunctional B cell activity frequently reported with exposure to Hg. Reduced concentrations of IgM have been associated with clinical disorders, including autoimmune diseases such as celiac disease and systemic lupus erythematosus (Manson *et al.,* 2005; Yel *et al.,* 2009). Class switching can be influenced by the cytokines available in the inflammatory milieu of the B-cell and the noted increased IL-10 in the Y19Hg cohort could potentially contribute to

increased class switching from IgM to other Ig subtypes (Tangye et al. 2002). Y19Hg was found to be significantly associated with higher CRP, IL-10 and a lower TNFα:IL-10. In normal healthy adults, such as this cohort, liver production of the acute phase protein CRP forms part of the innate immune response, for example Hg induced DAMPs (Pollard *et al*, 2018), which would be accompanied by a regulatory anti-inflammatory response via IL-10 induction and thereby suppressing TNF-α. IL-10 is associated with non-cell mediated immunity where IL-10 functions as a potent B cell stimulator that enhances activation, proliferation, and differentiation of B cells and may have a role in autoimmune disease through opposing the cellular mediated inflammatory Th17 response (Jorg *et al*., 2016). There is some evidence indicating that the anti-inflammatory actions of IL-10 are defective in autoimmune conditions with a noted increased IL-10 concentrations alongside reduced IL-10 receptor expression (Tournoy *et al*., 2000; Wang *et al*., 2017). In autoimmunity, elevated IL-10 has the potential to result in the persistent activation of autoreactive B cells and therefore exacerbate autoimmune disease where the normal immunoregulatory function of IL-10 is defective (Peng *et al*., 2013).

A recent examination of the Nutrition Cohort 2 (NC2) from the SCDS found that increasing MeHg was associated with decreasing Th1:Th2 (McSorley *et al.,* 2018). Hg modulation of cytokine and antibody responses may affect an individual's susceptibility to autoimmune type disease and also significantly alter host-pathogen interactions increasing susceptibility to infectious disease (Gardner *et al.,* 2010). Interestingly, at 19 years the n-3 LCPUFA were found to be associated with decreased CRP, INF-γ, TNFα, IL6, IL10 and with a higher TNFα:IL-10 ratio suggesting a regulatory effect on the immune system. Therefore, it is speculated where individuals are exposed to MeHg from fish consumption, the co-consumption of n-3 LCPUFA will prevent chronic inflammation and associated disease. A large observation study in Italy also reported that lower n-3 PUFA was associated with higher CRP and that higher n-3 PUFA was associated with lower IL-6, TNF-α and CRP (Ferrucci *et al.,* 2006). These results are supported by previous studies that have shown n-3 LCPUFA to have anti-inflammatory properties associated with reduced biomarkers of inflammation (Rangel-Huerta *et al.,* 2012, Pischon *et al.,* 2003; Ferrucci *et al.,* 2006; Calder 2015). Furthermore, interventions with n-3 LCPUFA have been shown to reduce disease activity and disease progression in a number of inflammatory conditions including autoimmune disease (Miles & Calder, 2012, Calder, 2013).

A strength of this research is the sizeable cohort who are high consumers of fish, have a wide range of hair MeHg (0.54 to 52.08 ppm) and a good status of n-3 LCPUFA as indicated by the low n6:n3 ratio. This longitudinal cohort has a low dropout rate and is well characterised across numerous timepoints including prenatally. The methods used in this

study to analyse MeHg exposure, PUFA status and markers of autoimmunity are considered to be highly sensitive in order to give the most precise results. Limitations of this study include its cross-sectional analysis and like all observational epidemiological studies no cause and effect can be determined. The analyses of this study focused on MeHg exposure and within the Seychelles it is believed that some 80% of hair THg is MeHg (Davidson *et al.,* 2004); however, this may vary among other populations (Ou *et al.,* 2014). Future studies should consider speciation of hair Hg or the use of Hg isotope ratios in hair in addition to total Hg concentrations to better assess exposure from fish derived MeHg (Sherman *et al.,* 2015). Genetic differences within individuals with respect to susceptibility to mercury-induced immune dysfunction (Gardner *et al.,* 2010) may explain differences in ANA concentrations within this and other cohorts. It is also important to remember that although ANA are used in the diagnosis and management of autoimmune disease their identification is not always associated with clinical disease and interpretation must be cautious. It would be important to also consider additional ANAs which have been previously linked with Hg exposure such as anti-fibrillarin autoantibodies and anti-glomerular basement membrane (Yang et al., 2001). Furthermore, blood samples were stored for circa 10 years before analyses which could affect cytokine measurements (Zhou *et al.,* 2010) and in part may explain the large number below the LOD and thereby reducing the percentage of positivity.

In summary, MeHg exposure at 19 years was associated with higher ANA and lower IgM but only following adjustment for LCPUFA which may suggest immune dysregulation. Total n-3 LCPUFA was associated with lower markers of inflammation. This study has global relevance given the importance of fish consumption as a source of protein and nutrition and that the global consumption of fish has reached an all-time high (FAO, 2018). Nevertheless, the clinical significance of these findings is unclear and further research is warranted to determine if these associations precede autoimmune disease development.

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Table 1: Participant characteristics (n=497)

	n	Mean	SD	Range
Male:female	229:268			
Waist circumference (cm)	490	75.86	10.48	52-118.20
Maternal hair MeHg (ppm)	*497*	*6.84*	4.55	0.54-26.73
Y19 hair MeHg (ppm)	448	10.23	6.02	0.42-52.08
Weighted average hair MeHg (ppm)	368	7.46	2.82	2.28-20.32
AntiCombined ANA (% >LOD)	473	56		
IgA (g/L)	471	1.84	0.61	0.37-3.88
IgG (g/L)	471	13.19	2.46	7.52-23.08
IgM (g/L)	471	1.23	0.62	0.26-4.48
Fish consumption (meals/week)	217	7.22	3.66	0-29
n-3 LCPUFA (mg/ml)	491	0.04	0.02	0.01-0.11
n-6 LCPUFA (mg/ml)	491	0.15	0.04	0.02-0.55
n-6:n-3 LCPUFA	491	3.81	1.97	0.96-17.73

MeHg, methylmercury; Y19, year 19; ppm, parts per million; ANA, antinuclear antibody; LCPUFA, long chain polyunsaturated fatty acids

Table 2. Main effect models reporting covariate-adjusted associations between methylmercury (MeHg) exposure and antinuclear antibodies (ANA) and immunoglubulins (Ig) and inflammatory markers with and without adjustment for long chain polyunsaturated fatty acids (LCPUFA)

		MatHg	Y19Hg
ANA combined[1]	Unadjusted	0.010 (□0.031, 0.051) p=0.637	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	0.017 (□0.028, 0.062) p=0.472	0.036 (0.001, 0.073) p=0.051
	Adjusted for n-3:n-6 LCPUFA	0.019 (□0.025, 0.064) p=0.395	**0.036 (0.001, 0.073) p=0.049**
Anti-dsDNA	Unadjusted	□0.002 (□0.045, 0.039) p=0.909	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	0.007 (□0.039, 0.053) p=0.750	0.010 (□0.026, 0.046) p=0.565
	Adjusted for n-3:n-6 LCPUFA	0.005 (□0.041, 0.050) p=0.826	0.010 (□0.026, 0.046) p=0.570
Anti-dsDNA[2]	Unadjusted	0.002 (-0.030, 0.034) p=0.908	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.004 (-0.040, 0.031) p=0.810	-0.010 (-0.037, 0.017) p=0.467
	Adjusted for n-3:n-6 LCPUFA	-0.002 (-0.037, 0.033) p=0.905	-0.010 (-0.037, 0.017) p=0.472
Anti-RNP A	Unadjusted	0.001 (□0.045, 0.046) p=0.950	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	□0.001 (□0.052, 0.048) p=0.969	□0.002 (□0.043, 0.036) p=0.921
	Adjusted for n-3:n-6 LCPUFA	0.009 (□0.041, 0.057) p=0.733	□0.001 (□0.041, 0.037) p=0.960
Anti-RNP A[2]	Unadjusted	-0.054 (-0.806, 0.699) p=0.888	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.015 (-0.827, 0.797) p=0.971	-0.003 (-0.625, 0.619) p=0.993
	Adjusted for n-3:n-6 LCPUFA	-0.154 (-0.960, 0.652) p=0.708	-0.014 (-0.639, 0.611) p=0.964
IgG	Unadjusted	□0.001 (□0.005, 0.002) p=0.435	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	□0.003 (□0.007, 0.001) p=0.162	□0.001 (□0.004, 0.002) p=0.609

	Adjusted for n-3:n-6 LCPUFA	□0.002 (□0.006, 0.002) p=0.261	□0.001 (□0.004, 0.002) p=0.639
IgM	Unadjusted	0.003 (□0.006, 0.012) p=0.459	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	0.002 (□0.008, 0.011) p=0.714	**□0.009 (□0.016, □0.002) p=0.016**
	Adjusted for n-3:n-6 LCPUFA	0.003 (□0.007, 0.012) p=0.557	**□0.009 (□0.016, □0.001) p=0.018**
IgA	Unadjusted	0.004 (□0.003, 0.011) p=0.274	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	0.004 (□0.004, 0.011) p=0.356	□0.002 (□0.008, 0.004) p=0.569
	Adjusted for n-3:n-6 LCPUFA	0.004 (□0.004, 0.011) p=0.342	□0.002 (□0.008, 0.004) p=0.562
Il-1 beta	Unadjusted	-0.002 (-0.007, 0.002) p=0.321	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.003 (-0.008, 0.002) p=0.241	0.002 (-0.002, 0.006) p=0.307
	Adjusted for n-3:n-6 LCPUFA	-0.003 (-0.008, 0.002) p=0.283	0.002 (-0.002, 0.006) p=0.303
IL-2	Unadjusted	-0.000 (-0.003, 0.003) p=0.951	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.000 (-0.004, 0.003) p=0.865	-0.000 (-0.003, 0.003) p=0.994
	Adjusted for n-3:n-6 LCPUFA	-0.000 (-0.004, 0.003) p=0.938	0.000 (-0.003, 0.003) p=0.985
IL-6	Unadjusted	0.014 (-0.007, 0.036) p=0.199	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.000 (-0.023, 0.022) p=0.970	0.007 (-0.010, 0.025) p=0.411
	Adjusted for n-3:n-6 LCPUFA	0.008 (-0.015, 0.031) p=0.485	0.007 (-0.010, 0.025) p=0.412
IL-10	Unadjusted	0.011 (-0.006, 0.028) p=0.200	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	0.001 (-0.018, 0.019) p=0.937	**0.016 (0.002, 0.030) p=0.027**
	Adjusted for n-3:n-6 LCPUFA	0.006 (-0.012, 0.025) p=0.514	**0.016 (0.002, 0.031) p=0.028**
INF-γ	Unadjusted	0.001 (-0.018, 0.020) p=0.902	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.008 (-0.029, 0.013) p=0.443	0.013 (-0.004, 0.029) p=0.125
	Adjusted for n-3:n-6 LCPUFA	-0.002 (-0.023, 0.020) p=0.888	0.013 (-0.003, 0.030) p=0.118
TNF-α	Unadjusted	-0.000 (-0.008, 0.008) p=0.995	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.005 (-0.014, 0.004) p=0.279	0.000 (-0.007, 0.007) p=0.958
	Adjusted for n-3:n-6 LCPUFA	-0.003 (-0.012, 0.006) p=0.555	0.000 (-0.007, 0.007) p=0.947
CRP	Unadjusted	-0.009 (-0.038, 0.021) p=0.564	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.014 (-0.044, 0.017) p=0.377	**0.031 (0.007, 0.054) p=0.011**

TNH-α: IL10 ratio	Adjusted for n-3:n-6 LCPUFA	-0.010 (-0.040, 0.020) p=0.517	**0.031 (0.007, 0.054) p=0.011**
	Unadjusted	-0.011 (-0.024, 0.002) p=0.091	
	Adjusted for n-3 LCPUFA, n-6 LCPUFA	-0.006 (-0.020, 0.009) p=0.436	**-0.016 (-0.027, -0.005) p=0.005**
	Adjusted for n-3:n-6 LCPUFA	-0.009 (-0.023, 0.005) p=0.223	**-0.016 (-0.027, -0.005) p=0.005**

Data presented as odds ration (95% confidence interval) p value. All values are log transformed.

MatHg, prenatal methylmercury exposure; Y19Hg, year 19 concurrent MeHg exposure

Unadjusted: controlled for sex of child, maternal SES, maternal age and MatHg.

Adjusted for n-3 LCPUFA, n-6 LCPUFA: controlled for sex of child, waist circumference (WC), MatHg and Y19Hg.

Adjusted for n-6:n-3 LCPUFA: controlled for sex of child, WC, MatHg and Y19Hg.

[1]ANA combined: within or above reference range for any of the 13 measured ANA [2] Anti-dsDNA and anti-RNP A were analyzed as dichotomous (<LOD or LOD+)

ANA; antinuclear antibody, dsDNA; double stranded DNA, RNP A; ribonuclear protein A, Ig; immuoglubulin, IL; interleukin, CRP; C reactive protein, INF-γ; interferon gamma, TNF-α; tumour necrosis factor alpha

Table 3. Associations between n-3 long chain polyunsaturated fatty acids (LCPUFA), n-6 LCPUFA and the n-6:n-3 LCPUFA ratio with immune markers controlling for maternal methylmercury (MatHg).

	n-3 PUFA	n-6 PUFA	n6:n3 ratio
ANA combined[1]	□1.148 (□15.030, 12.714) p=0.871	□2.380 (□7.166, 2.128) p=0.311	□0.021 (□0.125, 0.083) p=0.690
Anti dsDNA	9.343 (□4.888, 23.685) p=0.198	□1.019 (□5.966, 3.637) p=0.676	□0.059 (□0.175, 0.049) p=0.299
Anti RNP A	**□20.355 (□36.893, □4.336) p=0.014**	□1.442 (□6.652, 3.393) p=0.571	0.075 (□0.035, 0.185) p=0.173
CRP	**□13.805 (□23.155, □4.456) p=0.004**	0.451 (□2.627, 3.529) p=0.774	**0.091 (0.022, 0.161) p=0.010**
INF-γ	**□14.654 (□21.098, □8.211) p=0.000**	□1.559 (□3.680, 0.563) p=0.149	**0.054 (0.005, 0.103) p=0.030**
IgA	□0.221 (□2.586, 2.145) p=0.855	□0.058 (□0.824, 0.708) p=0.882	0.003 (□0.015, 0.020) p=0.753
IgG	□0.805 (□2.031, 0.420) p=0.197	□0.299 (□0.696, 0.097) p=0.139	0.000 (□0.009, 0.009) p=0.963
IgM	□1.215 (□4.156, 1.726) p=0.417	□0.423 (□1.376, 0.529) p=0.383	□0.007 (□0.029, 0.015) p=0.533
IL-1	0.236 (□1.387, 1.859) p=0.775	□0.377 (□0.912, 0.157) p=0.166	□0.005 (□0.017, 0.007) p=0.419
IL-10	**□18.461 (□24.125, □12.797) p<0.001**	0.560 (□1.305, 2.425) p=0.556	**0.110 (0.067, 0.153) p<0.001**
IL-2	0.007 (□1.125, 1.138) p=0.991	□0.133 (□0.506, 0.239) p=0.482	□0.003 (□0.011, 0.006) p=0.509
IL-6	**□23.841 (□30.760, □16.921) p<0.001**	□1.026 (□3.304, 1.252) p=0.377	**0.130 (0.077, 0.183) p<0.001**
TNF-α	**□7.541 (□10.275, □4.808) p<0.001**	0.196 (□0.704, 1.096) p=0.668	**0.045 (0.024, 0.065) p<0.001**
TNF-α:IL-10	**10.920 (6.549, 15.291) p<0.001**	□0.363 (□1.802, 1.076) p=0.620	**□0.065 (□0.098, □0.032) p<0.001**
Anti dsDNA[2]	□7.354 (□18.227, 3.518) p=0.184	0.523 (□2.990, 4.035) p=0.770	0.041 (□0.040, 0.122) p=0.322
Anti RNA[2]	**295.607 (44.831, 546.383) p=0.021**	28.539 (□52.488, 109.565) p=0.489	□1.085 (□2.963, 0.793) p=0.257

Models controlled for sex of child, maternal SES, maternal age and MatHg.

ANA; antinuclear antibody, dsDNA; double stranded DNA, RNP A; ribonuclear protein A, CRP; C reactive protein, INF-γ; interferon gamma, Ig; immuoglubulin, IL; interleukin, TNF-α; tumour necrosis factor-alpha,

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2, Anti-dsDNA and anti-RNP A were analyzed as dichotomous (<LOD or LOD+)

All values are log transformed.

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Manuscript Title

Methylmercury and long chain polyunsaturated fatty acids are associated with immune dysregulation in young adults from the Seychelles Child Development Study.

Highlights

1. Concurrent methylmercury (MeHg) exposure is associated with a higher odds ratio of being positive for the presence of an antinuclear antibody (ANA) and a lower IgM but only following adjustment of n-3 long chain polyunsaturated fatty acids (LCPUFA) or the n-6:n-3 LCPUFA ratio.
2. Prenatal methylmercury exposure was not associated with ANA or any biomarker of inflammation at age 19 years.
3. N-3 LCPUFA were associated with lower markers of inflammation whereas a higher n-6:n-3 LCPUFA ratio was associated with higher biomarkers of inflammation.
4. Further research is required to determine if MeHg exposure at an older age is associated with the onset of autoimmune disease.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: